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MINIREVIEW

The Bases of Crown Gall Tumorigenesis

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The nine decades since Smith and Townsend demonstrated that *Agrobacterium tumefaciens* causes plant tumors (95) have been marked by a series of surprises. Among the most important of these was the report in 1958 that these tumors could be excised and propagated in vitro without exogenous plant hormones (7). Equally important were a series of reports beginning about the same time that tumors released compounds that agrobacteria could use as nutrients (24). Perhaps the most exciting discoveries, reported in the 1970s and 1980s, were that tumorigenesis required the transfer of fragments of oncogenic DNA to infected plant cells (10), that this process evolved from a conjugal transfer system (99), and that the genes that direct this process are expressed in response to host-released chemical signals (47). This DNA transfer process has become a cornerstone of plant molecular genetics. The genus *Agrobacterium* also has provided excellent models for several aspects of host-pathogen interactions, including intercellular transport of macromolecules (11), bacterial detection of host organisms (47), targeting of proteins to plant cell nuclei (3), and interbacterial chemical signaling via autoinducer-type pheromones (120).

Most of the genes required for tumorigenesis are found on large extrachromosomal elements called Ti plasmids. Indeed, transfer of Ti plasmids into certain nonpathogenic bacteria converts them into tumorigenic pathogens (43). Ti plasmids are generally referred to by the types of opines whose catabolism they direct (see below). However, this nomenclature is becoming less satisfactory as we discover that all known Ti plasmids direct the catabolism of more than one opine and that opine catabolic genes are found in a variety of combinations in different plasmids. The Ti plasmids pTiA6NC, pTi15955, pTiAch5, pTiR10, and pTiB6S3, which are widely considered to be functionally identical, are generally referred to as octopine-type Ti plasmids (or, less frequently, octopine, mannityl opine-type Ti plasmids). The DNA sequencing of these plasmids was initiated almost 20 years ago (21) and was recently completed in our three laboratories. The resulting 194,140-nucleotide sequence is a composite assembly of sequences from all of the plasmids listed above. The close similarity of these plasmids is exemplified by the sequence of a 42-kb segment of the *vir* regions of pTiA6NC and pTi15955. These sequences differ at only one base, and this polymorphism is silent at the amino acid level. We have no evidence for polymorphisms elsewhere except for a large deletion that is unique to pTiA6NC (Fig. 1). The restriction map deduced from this sequence agrees almost perfectly with the published restriction map of pTiAch5 (25). All known and suspected genes are depicted in Fig. 1, and their demonstrated or putative functions are described in Table 1. The DNA sequence of this Ti plasmid provides a useful framework to review the roles of this plasmid in the biology of plant infection and colonization.

This Ti plasmid contains 155 open reading frames (ORFs), almost all of which are likely to encode functional proteins (Fig. 1 and Table 1). The overall G+C composition of this plasmid is 55%, although a few segments are considerably richer in A's and T's, particularly in the T region (see below). Overall, the Ti plasmid exhibits a modular structure with genes of similar function or purpose grouped together. Thus, we can define five components: (i) the T region, which codes for sequences that are transferred to the plant host; (ii) the *vir* region, which directs the processing and transfer of the T-DNA; (iii) the *rep* region, which is required for replication of the Ti plasmid; (iv) the *tra* and *trb* loci, which direct the conjugal transfer of the Ti plasmid; and (v) genes that direct uptake and catabolism of opines. An exception to this clustering is the *tra* and *trb* loci, the two gene sets required for conjugal transfer, which are separated from each other by 60 kb.

TRANSFER OF TWO DNA FRAGMENTS TO HOST PLANT CELLS

During infection, *A. tumefaciens* strains carrying an octopine-type Ti plasmid transfer two fragments of DNA to the nuclei of host plants by a mechanism that requires cell-cell contact and resembles plasmid conjugation. These fragments are designated the T_L-DNA and T_R-DNA (Fig. 1, top line), and are 13 and 7.8 kb in length, respectively (4, 105). The corresponding segments of the Ti plasmid are called T regions, and each is flanked by *cis*-acting, 25-bp direct repeats, called border sequences (121, 125). The left border of the T_L-DNA is dispensable for T-DNA transfer, while the right border is essential and acts in a polar fashion, suggesting that transfer may initiate at the right border and proceed leftward (76). Inversion of the right border leads to attenuated tumorigenesis, and tumors made by such mutants contain extremely long T-DNA fragments consisting of virtually the entire Ti plasmid (76). Adjacent to the right border of T_L is another *cis*-acting site called overdrive (94), which is required for wild-type transfer efficiency and provides a binding site for the VirC1 protein (see below). A second possible overdrive sequence is located adjacent to the right border of T_R, though the role of this sequence in T-DNA transfer has not been studied.

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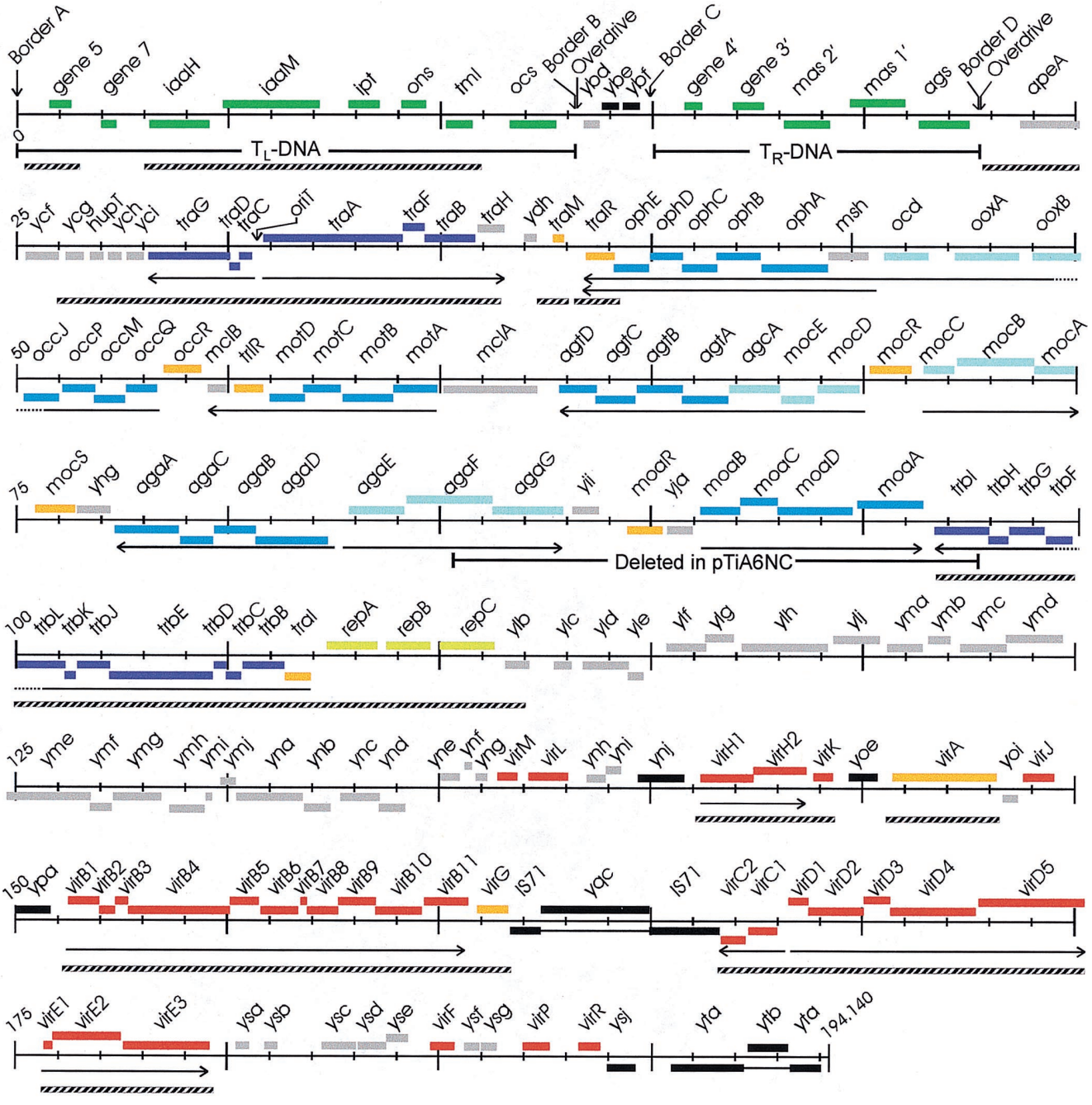

FIG. 1. Genetic map of the octopine-type Ti plasmid. Nucleotide 1 is taken as the left end of the left border of T_L. Genes transcribed from left to right are shown above the scale, while genes transcribed from right to left are shown below the scale. Known or suspected polycistronic operons are indicated with horizontal arrows. A 12.6-kb region deleted in the widely studied pTiA6NC is indicated by a horizontal bar on line 4. Regions that can form heteroduplexes with orthologous sequences of pTiC58 (27) are indicated with crosshatched bars. Green bars indicate genes that are located on the T_L region or T_R region and transferred to plant cells. Red bars indicate genes in the *vir* regulon, all of which are regulated by VirA and VirG, and many of which are required for T-strand processing and transfer. Purple bars indicate genes required for interbacterial conjugal transfer of the Ti plasmid. Light green bars indicate genes required for vegetative replication and partitioning of the Ti plasmid. Dark blue bars indicate genes encoding ATP binding cassette-type opine permeases, while light blue bars indicate opine catabolic genes. Orange bars indicate regulatory genes. Black bars indicate suspected IS elements (in this case, bars represent DNA sequence similarities rather than ORFs), while grey bars indicate ORFs of miscellaneous or unknown function. Gene names beginning with the letter "y" indicate the position of the gene, such that the second letter represents position in tens of kilobases, and the third letter indicates position in single kilobases. During the analysis of all these sequences, we found that *tml*, *mas2'*, *mas1'*, and *ags* had sequence discrepancies relative to orthologous genes from other Ti or Ri plasmids. These regions were resequenced after PCR amplification, and three errors in the original sequence (4) were detected and corrected. The resulting DNA sequence of the Ti plasmid has been deposited in the GenBank DNA sequence database (accession no. AF242881). Most Ti plasmid sequences were previously deposited in DNA sequence databases, and the original sources of these sequences are provided in the annotations of our compiled sequence.

TABLE 1. Genes encoded by the octopine-type Ti plasmid[a]

Genetic locus	Description	Reference(s)
T-DNA genes		
ags	Agropine synthase, lactonization of mannopine	24, 40
Gene *5*	Synthesis of indole-3-lactate, an auxin antagonist	57
iaaH and *iaaM*	Conversion of tryptophan to indole acetic acid (auxin)	55
ipt	Condensation of AMP and isopentenylpyrophosphate to form isopentenyl-AMP, a cytokinin	66
mas1′ and *mas2′*	Mannopine synthase; condensation of glucose with glutamine or glutamate followed by reduction	24
ocs	Octopine synthase, reductive condensation of pyruvate with four basic amino acids	21
ons	Opine export from plant cells	75
tml (gene *6b*)	Auxin sensitivity	108
Borders A, B, C, D	*cis*-acting sites required for T-DNA processing, functionally equivalent to conjugal origins of transfer	125
Overdrive	*cis*-acting site for optimal T-DNA transfer; VirC1 binding site	110, 113
vir loci		
virB1-11	Type IV transport system to transfer T-DNA and Vir proteins from bacteria to host cytoplasm	106, 116
virC and *-D*	T-DNA processing. VirD1 and VirD2 nick at T-DNA borders; VirC1 binds overdrive	111, 122, 123
virE	Nuclear transport of T-DNA. VirE2 binds single-stranded DNA and has nuclear localization sites; VirE1 is a chaperone for VirE2 transport	14, 101
virF	Host range factor	73
virH1-2	P450-type oxidases; VirH2 O demethylates phenolic inducers	51
virM, *-L*, *-K*, *-J*, *-F*, *-P*, *-R*, *-D3*, *-D5*, and *-E3*	Other members of the *vir* regulon; VirP resembles phosphatases	50, 52
Interbacterial conjugation genes		
traAFB, *traCDG*	Ti plasmid conjugal DNA processing	1
trbB-I	Type IV transfer system required for Ti plasmid conjugation	1
oriT	*cis*-acting site required for conjugation	1
Vegetative replication genes		
repAB	Putative partitioning system	104
repC	Essential for vegetative replication	104
Opine uptake genes		
agaDBCA	Agropinic acid permease	70
agtABCD	Agropine permease	Unpublished data
motABCD	Mannopine permease	80
moaBCDA	Mannopinic acid permease	70
occQMPJ	Octopine permease	112
ophABCDE	Putative permease for an unknown substrate	33
Opine catabolism genes		
agaE	Conversion of agropinic acid to mannopinic acid	70
agaFG	Conversion of mannopinic acid to glutamic acid and mannose	70
agcA	Catabolic mannopine cyclase, for conversion of agropine to mannopine, related to *ags*	54
mocAB	Oxidoreductase, and dehydratase?	54
mocCD	Conversion of mannopine to glutamine and glucose	54
mocE	Kinase?	54
ocd	Ornithine cyclodeaminase for conversion of ornithine to proline	114
ooxAB	Oxidoreductase for conversion of octopine-type opines to pyruvate and corresponding basic amino acid	114
Transcriptional regulation genes		
moaR	Repressor of *agaD-A*, *agaE-G*, and *moaB-A* operons	70
mocR	Probable regulator of the *mocD-agtD* and *mocC-A* operons	54
mocS	Resembles MocR, function unknown	54
occR	LysR-type octopine-responsive regulator of the *occQ-traR* operon	115
traR and *traI*	LuxR-LuxI-type quorum sensing regulators; TraI synthesizes 3-oxooctanoylhomoserine lactone; TraR is a transcriptional activator	36, 77, 128
traM	TraR antagonist	32
trlR	TraR antagonist; TrlR resembles TraR but is truncated and may inhibit TraR by forming inactive heteromultimers	80, 127
virA and *virG*	Two-component regulators of *vir* regulon; VirA is a transmembrane histidine kinase; VirG is an OmpR-type response regulator	61
IS elements		
IS71L and IS71R	Apparent IS element, interrupted by insertion of *yqc* IS element	Unpublished data
ybe, *yoe*, and *yqc*	Resemble IS*66* of *A. tumefaciens*	Unpublished data
ybf	Resembles IS*1203* of *E. coli*	Unpublished data
ynj	Resembles IS*1313* of *A. tumefaciens*	Unpublished data
ypa	Resembles IS*869* of *A. tumefaciens*	Unpublished data
ysj	Resembles IS*492* of *Pseudomonas* sp.	Unpublished data
yta	Resembles IS*21* of *E. coli*, disrupted by IS element *ytb*	Unpublished data
ytb	Resembles IS*1111*a of *Coxiella burnetii*	Unpublished data

Continued on following page

TABLE 1—*Continued.*

Genetic locus	Description	Reference(s)
Genes with miscellaneous and unknown functions		
apeA	Exclusion of bacteriophage Ap1	1
hupT	Resembles HNS-type proteins	1
mclA and *-B*	Resembles methyl-accepting chemotaxis proteins; possible role in chemotaxis to opines. MclB is severely truncated and inhibits chemotaxis in *A. tumefaciens*	80, 127
msh	Resembles methionine synthase	33
yhg	Resembles oxidoreductases; possible role in opine catabolism	70
ylb	Resembles DNA invertases; possible role in plasmid maintenance	Unpublished data
yld	Resembles DNA invertases; possible role in plasmid maintenance	Unpublished data
yle	Resembles plasmid stability locus; possible role in plasmid maintenance	Unpublished data
ylf-yng	Functions unknown	Unpublished data
ysa	Resembles integration host factor; weakly induced by *vir* inducing stimuli	52
ysb	Resembles cold shock proteins	52
ysc, *ysd*, and *yse*	*ysc* and *ysd* resemble 3′ end of *traA* of Ti plasmid; *yse* resembles Ti plasmid *traF*; none is detectably expressed	29, 52

[a] Types of genes correspond to bars in Fig. 1 as follows: T-DNA genes, dark green bars; *vir* loci, red bars; interbacterial conjugation genes, purple bars; vegetative replication genes, light green bars; opine uptake genes, dark blue bars; opine catabolism genes, light blue bars; transcriptional regulation genes, orange bars; IS elements, black bars; and genes with miscellaneous and unknown functions, grey bars.

In the presence of proteins encoded by the *vir* region (see below), the DNA within the T regions undergoes several processing steps (Fig. 2). Each border is cleaved on the bottom DNA strand at a site exactly 4 nucleotides from its left end. This reaction is catalyzed by the VirD2 protein (see below), which remains covalently bound to the 5′ end of each cleaved strand. While the top strand remains in duplex form, approximately half of the bottom strands can be recovered in a single-stranded linear form, referred to as T strands (97). These T strands are thought to represent the transferred form of the T-DNA and are probably formed by displacement during rolling-circle DNA synthesis that initiates from the 3′ ends of each right border. At an early stage of transformation, T strands can be detected in plant cells (124), showing that the T-DNA is transferred in a single-stranded form. T strands are integrated into the host genome at apparently random sites by illegitimate recombination (72) and are stably transmitted to daughter plant cells upon mitotic cell division, and during meiosis and syngamy.

EXPRESSION AND FUNCTIONS OF TRANSFERRED GENES

Collectively, T_L-DNA and T_R-DNA encode 13 proteins (Fig. 1, dark green bars). The nontranscribed regions of each transferred gene possess many of the features of plant genes, including typical eukaryotic TATA and CAAT boxes, transcriptional enhancers, and poly(A) addition sites (6). No introns have been reported for any of the *A. tumefaciens* transferred genes, although at least one T-DNA gene in *Agrobacterium rhizogenes* contains an intron in its 5′ nontranslated region (71). The coding regions of the T-DNAs have a G+C content of approximately 50%. However, the intergenic regions, especially the 3′ nontranslated regions, are extremely poor in G's and C's, approximately 20 to 30%.

One group of T-DNA genes directs the production of plant growth hormones that are responsible for the proliferation of the transformed plant cells (6). The *iaaM* and *iaaH* products direct the conversion of tryptophan via indoleacetamide to indoleacetic acid (auxin). The *ipt* product condenses isopentenyl pyrophosphate and AMP (6), and host enzymes are presumed to convert the resulting isopentenyl-AMP into the cytokinin zeatin by removal of the phosphoribosyl group and hydroxylation of one methyl group of the isopentenyl moiety. Two other T-DNA genes are thought to play ancillary roles in tumorigenesis. The gene *5* product directs the synthesis of indole-3-lactate, an antagonistic auxin analogue (57), while *tml* (also designated gene *6b*) increases the sensitivity of plant cells to phytohormones by a mechanism that remains to be discovered (108). This gene can provoke tumors in certain host plants in the absence of the other oncogenes (42).

A second set of transferred genes directs the production of bacterial nutrients called opines. Octopine-type Ti plasmids direct their hosts to synthesize at least eight opines. The *ocs* gene encodes octopine synthase, which reductively condenses pyruvate with either arginine, lysine, histidine, or ornithine to produce octopine, lysopine, histopine, or octopinic acid, respectively, all of which can be detected in crown gall tumors (24). The *mas2′* product is thought to condense glutamine or glutamic acid with glucose (although this has not been experimentally demonstrated), while the *mas1′* product reduces these intermediates, forming mannopine and mannopinic acid, respectively. The *ags* product catalyzes the lactonization of mannopine to form agropine. Mannopine and agropine also can spontaneously lactamize to form agropinic acid (24). Thus, tumors induced by strains harboring octopine-type Ti plasmids can produce as many as four members of the octopine family and four members of the mannityl opine family.

Ti PLASMID-ENCODED PROTEINS REQUIRED FOR T-DNA PROCESSING AND TRANSFER

Proteins responsible for T-DNA processing and transfer are encoded by the *vir* region of the Ti plasmid. Twenty genes in this region are essential for wild-type levels of pathogenesis on most host plants and are expressed in six operons, *virA*, *-B*, *-C*, *-D*, *-E*, and *-G*. The proteins required for border cleavage are encoded by *virD1* and *virD2*, with the VirD2 protein remaining covalently bound to the 5′ end of the T-strands (98, 123). Purified VirD2 cleaves single-stranded oligonucleotides containing border sequences at the same site, creating a covalent bond between the 5′ phosphate and tyrosine 29 (86). This reaction is fully reversible, indicating that the DNA-protein phosphodiester linkage is a high-energy bond and suggesting that a reverse reaction might be important for the integration of T-DNA into the plant genome (109). VirD2 alone was not able to cleave the same sequence in double-stranded form but was able to do so in the presence of VirD1 (90). VirC1 binds to the overdrive site, which lies adjacent to the left border (111). VirC1 and VirC2 are not required for T-region process-

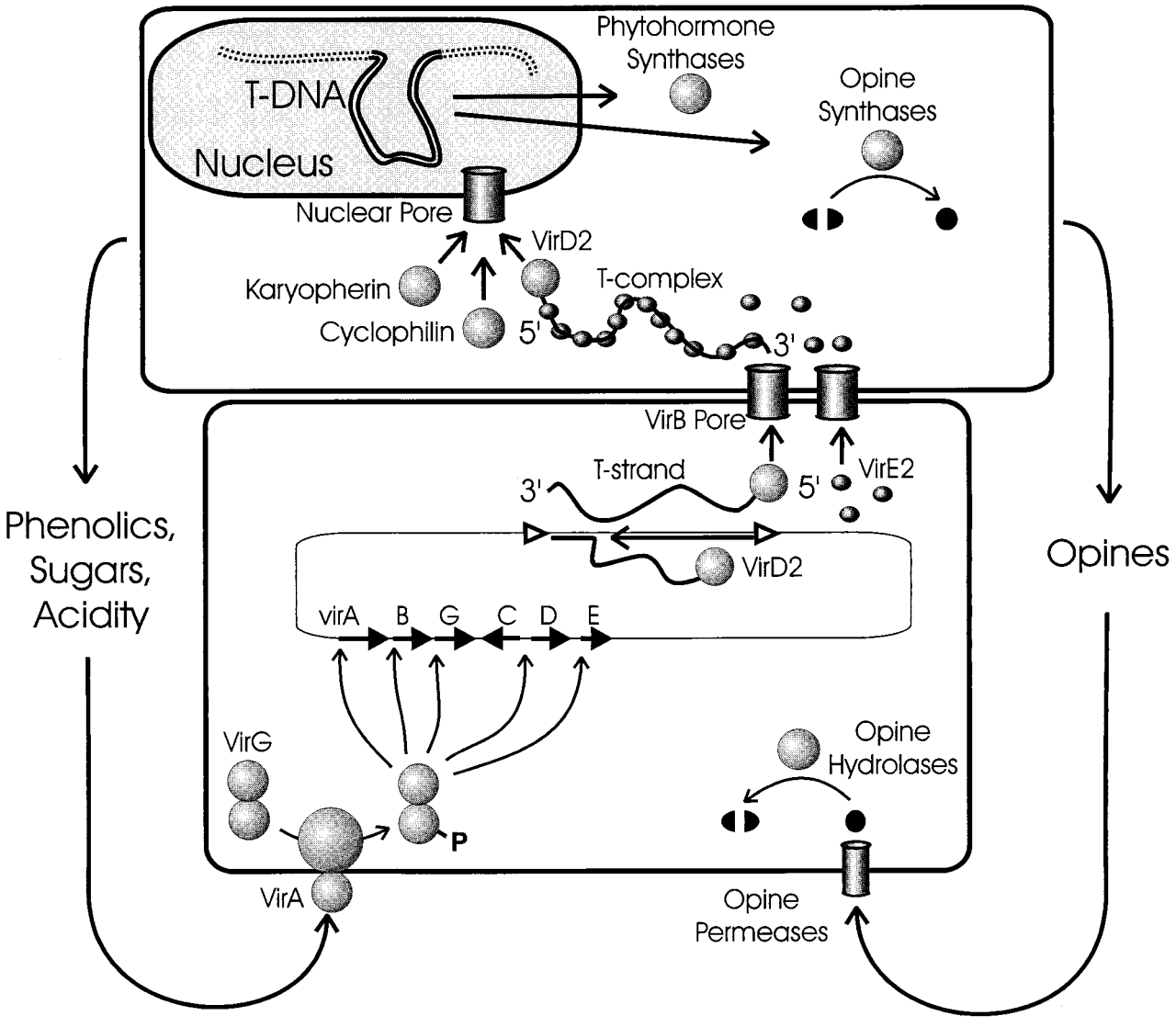

FIG. 2. Two-way exchange of chemical signals between *A. tumefaciens* and host plants. Wound-released chemical stimuli are perceived by the VirA to VirG proteins, which leads to transcription of *vir* promoters. T-DNA is processed by the VirD2 protein, and single-stranded linear T strands are formed by strand displacement. T strands and VirE2 are translocated from the bacteria via a pore encoded by the *virB* operon and form a T complex within the plant cytoplasm. T complexes are transported into the nucleoplasm via the host protein karyopherin alpha, and the T-DNA is integrated into genomic DNA. Transferred genes encode phytohormone synthases that lead to plant cell proliferation and opine synthases that provide nutrients to the colonizing bacteria. Opines are released from the plant cell, enter the bacteria via dedicated opine permeases, and are catabolized via opine-specific catabolic proteins. Opine permeases and catabolic enzymes are encoded by the Ti plasmid. For the sake of clarity, the relative orientations of *vir* genes and T-DNA have been inverted.

ing but are required for efficient T-strand transfer into most host plants, suggesting that they play a role in T-strand export.

The T-DNA transfer apparatus is encoded by the *virB* operon, which contains 11 genes (11). Each except VirB1 is essential for tumorigenesis (5). All 10 essential proteins have been localized to the inner or outer membrane, and most appear either to be integral membrane proteins or to be exported from the cytoplasm (107). Two VirB proteins, VirB4 and VirB11, are peripherally bound to the others and located primarily in the cytoplasm, although a small part of VirB4 may span the inner membrane (19). VirB4 and VirB11 have ATPase activity and are thought to provide the energy required for export of other protein subunits, for T-strand transport, or both (12, 93). VirB proteins direct the production of pili that resemble conjugative pili (31), and VirB2 is the major subunit of these pili (58). VirB2 is processed to a 7.2-kDa product that is cyclized such that the amino terminus is linked to the carboxyl terminus via an amide bond (26). Cyclization does not require any Ti plasmid-encoded proteins but does not occur in *Escherichia coli*, suggesting that this reaction requires a protein encoded elsewhere in the *A. tumefaciens* genome. VirB7 may help to anchor this pilus to the bacterial cell, as it is an outer membrane lipoprotein that forms disulfide bonds with the periplasmically localized VirB9 (2, 96). The VirB mating bridge is thought to be coupled to the T-strand complex by the VirD4 protein, which is located in the inner membrane and is absolutely required for transfer (67, 82). VirB1 possesses sequence motifs found in bacterial transglycosylases and eukaryotic lysozymes, suggesting a role in the localized digestion of the peptidoglycan (78).

The VirB apparatus delivers T strands to the plant cell cytoplasm, where additional steps are required to transport

this DNA to the nucleoplasm and to integrate it into host DNA. The carboxyl terminus of VirD2 contains a nuclear localization signal that is thought to guide nuclear targeting by interacting with the karyopherin alpha and cyclophilin proteins (3, 23, 44). The VirE2 protein appears also to play a role in nuclear import. This protein binds tightly and cooperatively to single-stranded nucleic acids, forming coiled, cylindrical filaments (14). Like VirD2, VirE2 contains nuclear localization sites that mediate transport of the T-DNA from the cytoplasm to the nucleoplasm (15).

Transgenic plants expressing VirE2 can be transformed by *virE2* mutants of *A. tumefaciens*, indicating that VirE2 is required only in plant cells (15). Similar data have been obtained for another protein, VirF, which is required for tumorigenesis on such plants as tomato and *Nicotiana glauca* (87). Mutations in either *virE2* or *virF* can be complemented extracellularly, that is, by coinfection with a helper strain possessing the *vir* region but lacking an oncogenic T-DNA (73, 84). Initially, it was thought that such complexes were formed within the bacterium, but more recent genetic evidence suggests that VirE2 and T-DNA are transferred separately and form complexes in the plant cell cytoplasm (101). Transfer of VirE2 requires VirE1, while transfer of T-DNA does not, suggesting that VirE1 acts as an export chaperone for VirE2 (22, 101). Conversely, transfer of T-DNA requires VirC1 and VirC2 while transfer of VirE2 does not require either protein (13). These studies provide the best evidence that T strands and VirE2 are transferred independently, although biochemical evidence addressing this hypothesis will await future studies. These data indicate that the *virB*-encoded transfer system, in addition to transferring T-DNA, can carry out contact-dependent translocation of at least three proteins, VirD2, VirE2, and VirF. This property of protein transport is highly reminiscent of the family of type III protein translocation systems of plant and animal pathogens, although these systems have independent ancestries (18, 37).

Many aspects of T-DNA transfer resemble interbacterial conjugal transfer of plasmid DNA (63). In both processes, transfer is initiated by single-stranded scissions at specific *cis*-acting sites. Moreover, the protein that catalyzes the scission remains bound to the 5′ end of the cleaved strand, and in both cases, DNA is transferred in a single-stranded form. The most direct evidence that the T-DNA transfer apparatus evolved from a conjugal transfer system is the extensive sequence similarities between Vir proteins and certain Tra proteins. For example, all 11 VirB proteins resemble the mating pair formation (Mpf) subset of Tra proteins encoded by the IncN plasmid pKM101 and show a lower degree of similarity to the Tra proteins of IncW, IncP, and IncF plasmids (48, 62). Similarly, the VirD1, VirD2, and VirD4 proteins resemble the donor transfer and replication (Dtr) subset of Tra proteins. In fact, the *virB* and *virD* operons together would constitute a complete set of conjugation proteins. The T-DNA border resembles the *oriT* sites of IncP plasmids, and nicking occurs at identical positions in the two transfer systems (117). The gene family of Vir and Tra proteins also includes the Ptl proteins of *Bordetella pertussis*, which direct the export of pertussis toxin; the VirB proteins of *Brucella* spp., which are required for intracellular survival; and other protein translocators of bacterial pathogens, collectively referred to as type IV export systems (18). The VirC and VirE proteins do not significantly resemble known transfer proteins, and VirC1 resembles a plasmid partitioning protein (38).

Some members of the *vir* regulon are not essential for tumorigenesis on all hosts and may be required only in specific hosts or may play other roles in pathogenesis. These include *virD5*, *-E3*, *-F*, *-H*, *-J*, *-K*, *-L*, *-M*, *P*, and *-R* (50, 52). However, the lack of an apparent role in tumorigenicity could be a consequence of functional redundancy. For example, *virJ* is essential for tumorigenicity, but only in the absence of the homologous chromosomal gene *acvB* (49). The *virH* operon consists of two genes whose products resemble the family of P450 monooxygenases (51). VirH2 chemically modifies certain phenolic *vir* gene inducers by O demethylation, converting them to noninducers (51). For example, the inducer ferrulic acid is O demethylated to create the noninducer caffeic acid. This finding suggests that VirH2 acts as a regulatory governor.

UPTAKE AND CATABOLISM OF OPINES

As described above, several T-DNA-encoded genes direct the synthesis of opines, which serve the bacteria as nutrient sources. Over 40 genes are devoted to opine uptake and catabolism. These include no fewer than six ATP binding cassette-type permeases (Fig. 1, dark blue bars) and 12 opine catabolic enzymes (light blue bars), whose functions are summarized in Table 1. These opine permeases are only distantly related to each other, suggesting that they were adapted from diverse sources. An additional gene (*mclA*) could encode a protein that resembles methyl-accepting chemoreceptors. *A. tumefaciens* strains are chemotactic toward opines, and chemotaxis requires the cognate periplasmic opine binding proteins (each a component of an opine uptake system) but does not require Ti plasmid-encoded methyl-accepting chemotaxis proteins (53). It seems likely that this is another example of redundancy in which these periplasmic binding proteins can interact either with chromosomally encoded or with Ti plasmid-encoded methyl-accepting chemotaxis proteins.

Characteristically, Ti plasmids code only for the opine catabolism systems that correspond to the set of opine biosynthesis genes located in the T regions. This presents the interesting problem of how these paired gene systems arise and how they remain grouped together despite the fact that they are located in different segments of the plasmid. Sequence analysis of the mannopine-agropine catabolic loci indicated that certain of these genes resemble the cognate opine biosynthetic genes. The catabolic protein AgcA, which interconverts mannopine and agropine, resembles the agropine synthase protein Ags, which carries out the same reaction. In fact, *ags* can complement an *agcA* mutant for catabolism of agropine (40). Similarly, MocC and MocD, which together degrade mannopine, resemble Mas1′ and Mas2′, which synthesize mannopine (54). Based on these comparisons, we have suggested that the T-region genes coding for mannityl opine synthesis by the transformed plant cells arose by gene duplication from bacterial genes required for catabolism of these or closely related substrates (54). However, not all opine synthases resemble their corresponding catabolic enzymes. For example, the octopine and nopaline synthases do not resemble their cognate catabolic enzymes.

REPLICATION FUNCTIONS

A DNA fragment containing just *repA*, *repB*, and *repC* provides all functions required for stable replication in *A. tumefaciens* (104). Only *repC* is critical for vegetative replication, while *repA* or *repB* is required for stable plasmid inheritance. RepA and RepB resemble a family of plasmid partitioning systems that are thought to ensure that during cell division each daughter cell inherits at least one copy of the plasmid. All three genes resemble replication genes of other large, low-copy-number plasmids present in members of the family *Rhi-*

zobiaceae (64). Incompatibility functions also are determined by the DNA fragment containing *repABC* (56). The octopine-type Ti plasmid is incompatible with nopaline-type Ti plasmids (41), but in spite of the relatedness of their replicators, the octopine Ti plasmid is compatible with Ri plasmids (17).

INTERBACTERIAL CONJUGATION OF Ti PLASMIDS

The octopine Ti plasmid is capable of interbacterial conjugation (28) and contains a complete transfer system (Fig. 1, purple bars). On the basis of similarity to other conjugation systems, the cluster of *tra* genes probably is required for DNA transfer and replication, while the *trb* gene cluster is probably required for mating pair formation and could direct the synthesis of conjugal pili. In the closely related conjugation system of pTiC58, *traB* is not essential for transfer, although it is required for maximal efficiency, while *traH* is not required for efficient transfer (29) and is here designated a *tra* gene simply because it lies in the *tra* regulon (see below). All other *tra* and *trb* genes of pTiC58 are known or thought to be required for efficient conjugation (29, 65), with the exception of *trbK*, which is probably not required for conjugation but may mediate entry exclusion (28). The three operons of the conjugal transfer system are strongly conserved among all of the Ti plasmids analyzed to date (although one report [103] claimed otherwise, that study was based upon an incorrect DNA sequence). These genes also resemble the *tra* genes of at least one symbiosis megaplasmid, pNGR234a of *Rhizobium* sp. strain NGR234 (30). The Tra system functions independently of the T-DNA transfer system described above (16).

The *tra* genes appear to have diverse origins. TraG, TraF, and all 11 Trb proteins closely resemble IncP-type Tra proteins. In contrast, TraA, the putative nickase-helicase of this system, does not closely resemble any IncP-type Tra protein. Instead, the amino-terminal domain of TraA, which should contain *oriT* nicking activity, resembles the strand transferase of the IncQ plasmid RSF1010, while its carboxyl-terminal domain, which contains a possible helicase, resembles Tra proteins of IncN, IncW, and IncF plasmids. The *oriT* also resembles the corresponding site in RSF1010. Interestingly, the Vir system seems also to have chimeric origins, as all 11 VirB proteins resemble IncN Tra proteins, while two VirD proteins, VirD2 and VirD4, resemble IncP-type Tra proteins. The T-DNA borders resemble the *oriT* site of IncP plasmids (117). In all cases, sequence similarities between Ti plasmid Tra proteins and corresponding Vir proteins are relatively weak.

REGULATED EXPRESSION OF Ti PLASMID-ENCODED GENES

Virtually all of the genes described above are tightly regulated by proteins that also are encoded on the Ti plasmid (Fig. 1, orange bars). For example, the *vir* regulon is coordinately induced in response to host-released phenolic compounds in combination with monosaccharides and extracellular acidity in the range of pH 5.0 to 5.5 (47). This acidity may be necessary to protonate phenolic compounds, which would increase their membrane permeability. These chemical stimuli are detected by the transmembrane two-component sensor kinase VirA, which phosphorylates the response regulator VirG. Phospho-VirG positively regulates all *vir* promoters, including those of *virA* and *virG*, which results in positive autoregulation of this regulon (100, 119).

VirA contains four functional domains, designated the periplasmic, linker, kinase, and receiver domains (9), and exists as a dimer both in the presence and in the absence of inducing stimuli (85). The periplasmic domain is required for detection of a sugar binding protein called ChvE (8, 92), while the linker domain is required for detection of phenolic compounds, and the receiver plays an inhibitory role in *vir* gene expression (9). VirA can undergo autophosphorylation in vitro and transfers its phosphoryl group to Asp52 of VirG (46). The carboxyl-terminal domain of VirG binds to sequences called *vir* boxes that are found near all VirG-regulated promoters (88, 118). While there is still some controversy about whether phenolic inducers bind directly to VirA (59), genetic evidence suggests that this is so, since *virA* genes from different strains of *A. tumefaciens*, when introduced into an isogenic background, encode proteins that are stimulated by different types of inducers (60).

All opine uptake and catabolic systems are induced by their cognate substrates. For example, octopine induces transcription of a 14-kb *occQ-traR* operon via the OccR protein, a LysR-type regulator. OccR binds to its binding site, which lies directly upstream of the *occQ* promoter, in the presence or absence of octopine but undergoes a conformational change in response to octopine (115). The mannopine and agropine permeases and catabolic enzymes also are induced by the cognate opines, probably via the MocR protein, which resembles the LacI repressor of *E. coli*. Similarly, regulated expression of the *aga* and *moa* genes by the cognate opines requires the MoaR repressor, which resembles yet another family of regulators, including the galacticol repressor of *E. coli* (70). Expression of the opine catabolism gene sets also is influenced by global control systems. Transcription of the mannityl opine catabolism genes, while inducible by their cognate substrates, also is controlled by catabolite repression (39, 127), since these genes are not induced by mannopine or agropine when favored carbon sources such as glutamate or succinate also are provided. Furthermore, these catabolic genes are part of the nitrogen assimilation regulon, since catabolite repression by succinate is not observed when mannopine is provided as the sole source of nitrogen (39).

The TraR-TraI system positively controls expression of the *tra* and *trb* operons (Fig. 1, purple bars). Transcription of this regulon is controlled by a regulatory cascade that is initiated by octopine acting through OccR, which leads to expression of *traR* (33). TraR in turn is a direct positive regulator of the *tra* and *trb* genes (36). TraR is a member of the LuxR family of quorum-sensing transcriptional regulators (35), and its activity requires *N*-3-oxooctanoyl-L-homoserine lactone (126). Synthesis of this compound, called an autoinducer, is directed by the TraI protein, which utilizes 3-oxooctanoyl-acyl carrier protein and *S*-adenosylmethionine as substrates (36, 77). This compound is synthesized in the bacterial cytoplasm but diffuses across the cell envelope and acts as a bacterial pheromone, providing a mechanism for the bacteria to estimate their population densities (35). Since the Ti plasmid encodes both TraI and TraR, each conjugal donor takes a census of other donors rather than of recipients (34). Purified TraR binds one molecule of this compound per protein monomer and binds directly to dyad symmetrical DNA sequences called *tra* boxes, which are found directly upstream of the *traA*, *traC*, and *traI* promoters (34, 128). TraR stimulates transcription of *tra* promoters in vitro on supercoiled templates but is largely inactive on linear templates (128). DNA binding by TraR requires the autoinducer (68).

TraR activity is antagonized by two proteins encoded by the *traM* and *trlR* genes. Interestingly, the *traM* gene is positively regulated by TraR, thereby creating a negative autoregulatory loop (32). TraM is an antiactivator and directly interacts with the carboxyl terminus of TraR (45, 69). This interaction rapidly

inhibits TraR activity and disrupts TraR-DNA complexes (69). TrlR is very closely related to TraR in its autoinducer binding domain but lacks a DNA binding domain (80, 127) and is thought to form inactive heterodimers with TraR. The *trlR* gene is positively regulated by mannopine. Consistent with this, mannopine inhibits *tra* gene expression, while inhibition is abolished by a *trlR* mutation (80, 127).

INSERTION SEQUENCES (ISs) AND UNCHARACTERIZED ORFS

A number of possible ISs are present on the Ti plasmid (Fig. 1, black bars), although transposition of these elements has not been detected experimentally. Three of these resemble IS*66*, which was originally found inserted in the *iaaH* gene of a strain of an *A. tumefaciens* mutant that causes shooty teratomas rather than tumors. However, several of these IS*66*-like elements are considerably shorter than IS*66*, suggesting that they may be defective remnants of the original element. The other possible IS-like elements resemble a wide variety of IS elements in many eubacteria.

While most of the genes in the Ti plasmid have been ascribed functions, a contiguous 24-kb region (coordinates 112 to 136) contains 25 ORFs that have no known function (Fig. 1, grey bars). Most of the ORFs in this region are at least 100 codons in length and have moderately strong translation initiation motifs, and many of these ORFs appear to be translationally coupled to adjacent ORFs. All of these considerations suggest that these ORFs are expressed genes, but the functions of their products are at present unknown. Approximately half of these genes resemble genes identified in genome sequencing projects, though none of these homologous genes has been characterized genetically or biochemically.

RELATION OF THE OCTOPINE-TYPE Ti PLASMIDS TO OTHER PLASMIDS OF THE *RHIZOBIACEAE*

The modular structure of this Ti plasmid is entirely in keeping with the model presented by Otten and colleagues in which these elements evolve by IS-mediated intramolecular rearrangements and by recombination with other plasmids (83). Signs of such recombination events are scattered over the length of this plasmid. For example, *trlR* may well have arisen by a recombination event that fused the mannityl opine catabolism region with its attendant mannopine-regulated *traR* allele to the region just upstream of the octopine catabolism locus (80, 127). Similarly, the structural and regulatory association of the functional *traR* allele with the *occ* operon arose from a fortuitous recombination event that fused *traR* to *occ*. Interestingly, such associations of *traR* with various opine catabolism operons are a consistent feature of Ti plasmids (28, 33, 80, 127).

Despite this plasticity, certain gene associations seem to be strongly conserved among these elements. Most notably, the *repABC* complex is tightly linked with the *trb* operon in all Ti and opine catabolism plasmids that have been examined to date (pTiC58, pTi-SAKURA, and pAtK84b) (64). This conservation extends to at least four plasmids present in members of the genus *Rhizobium* (64), suggesting that this linkage is strongly selected. Consistent with this interpretation, the intergenic region separating the two divergently oriented gene systems on many of these plasmids, including all Ti plasmids, contains two copies of the *tra* box sequence. Coupled with the recent observation that copy number of the nopaline-type Ti plasmid is positively enhanced by TraR in a quorum-dependent fashion (64), it is reasonable to conclude that conjugal transfer and plasmid replication are functionally linked.

CONCLUSIONS

As described above, most of the genes of the Ti plasmid play direct or indirect roles in some aspect of tumorigenesis or tumor colonization. We understand the roles of most of the T-DNA-encoded genes, although the functions of some remain mysterious. We have some insights about the processing and transfer of the T-DNA, although our understanding of the VirB-encoded pore is rudimentary, as are the steps involved in nuclear transport and integration. At least three Vir proteins are thought to be transferred from the bacterium into plant cells during infection, though the physical detection of these proteins in plant cells remains a goal for future studies. It will be interesting to identify any additional translocated proteins and to elucidate their functions. VirA and VirG remain important paradigms for host detection, and the multidomain structure of VirA remains fertile ground for future work. Future studies will decide once and for all whether VirA binds phenolic inducers directly or through an accessory phenolic binding protein. Of the 34 known VirG-regulated genes, one-third do not seem essential for tumorigenesis (at least on certain host plants), suggesting that plant-released *vir*-inducing signals elicit multiple bacterial responses that remain to be described.

Another challenge lies in comparative analysis of the many different Ti plasmids that have been isolated, as well as other plasmids found in members of the *Rhizobiaceae*. We know that approximately 65 kb of octopine-type plasmids are conserved in the nopaline-type Ti plasmid pTiC58 (Fig. 1, crosshatched boxes), including part of the T-DNA and the *tra*, *trb*, *rep*, and *vir* regions (27), while the remaining 130 kb are not conserved. As more Ti plasmids and related plasmids are characterized (102), it will be possible to refine our insights about the evolution of these genetic elements.

The use of *A. tumefaciens* to create transgenic plants has become routine for many dicots as well as for some monocots, and yet new insights about fundamental aspects of *Agrobacterium*-plant interactions will lead to improved technologies in plant transformation. Future work will lead, for example, to further expansion of the organism's host range, to new approaches to transferring extremely long fragments of DNA, and to new approaches to using T-DNA to disrupt plant genes.

It is striking that such a large portion of the Ti plasmid is devoted to opine uptake and catabolism, and few of these systems have been studied in any depth. Studies of opine chemotaxis, uptake, and catabolism will continue. In addition, one challenge for the next 10 years will be to apply these insights about opines to agriculture. Several reports have already appeared showing that bacteria that utilize a particular opine enjoy a competitive advantage in colonizing transgenic plants that produce the same opine (81, 89). We suspect that this technology may revolutionize efforts to foster beneficial plant-microbe associations.

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